Human Prostate Cell Lines In Cancer Treatment

Field of the Invention

The invention relates to the treatment of primary, metastatic, and residual cancer in mammals, and more particularly to the use of materials such as whole cells and derivatives and portions thereof to stimulate the immune system to attack cancer.

Background to the Invention

Cancerous cells contain numerous mutations that can result in recognition of the cells by a host's immune system. Appreciation of this phenomenon has prompted much research into potential immunotherapies to harness the host's immune system for attacking cancer cells. Eliminating these cells or reducing them to a level that is not life-threatening has been a major goal, as reviewed in Maraveyas, A. & Dalgleish, A.G. 1997 *Active immunotherapy for solid tumours in vaccine design* in The Role of Cytokine Networks, Ed. Gregoriadis *et al.*, Plenum Press, New York, pages 129-145; Morton, D.L. and Ravindranath, M.H. 1996 *Current concepts concerning melanoma vaccines* in Tumor Immunology – Immunotherapy and Cancer Vaccines, ed. Dalgleish, A.G. and Browning, M., Cambridge University Press, pages 241-268.

Such work in the cancer immunotherapy field can be classified into five categories, non-specific immunotherapy, antibodies and monoclonal antibodies, subunit vaccines, gene therapy, and cell-based vaccines.

Non-specific immunotherapy

Efforts to stimulate the immune system non-specifically date back over a century to the pioneering work of William Coley (Coley, W.B., 1894 Treatment of inoperable malignant tumours with toxins of erisipelas and the Bacillus prodigosus. Trans. Am. Surg. Assoc. 12: 183). Although successful in a limited number of cases (e.g. BCG (i.e. bacille Calmette-Guérin) for the treatment of urinary bladder cancer, IL-2 for the treatment of melanoma and renal cancer) it is widely acknowledged that non-specific immunomodulation is unlikely to

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prove sufficient to treat the majority of cancers. While non-specific immune-stimulants may lead to a general enhanced state of immune responsiveness, they lack the targeting capability and also subtlety to deal with tumour lesions which have many mechanisms and plasticity to evade, resist and subvert immune-surveillance.

Antibodies and monoclonal antibodies

Passive immunotherapy in the form of antibodies, and particularly monoclonal antibodies, has been the subject of considerable research and development as anti-cancer agents. Originally hailed as the magic bullet because of their exquisite specificity, monoclonal antibodies have failed to live up to their expectation in the field of cancer immunotherapy for a number of reasons, (thereby abrogating their activity) including immune responses to the antibodies themselves and inability of the antibody to access the lesion through the blood vessels. To date, few products have been registered as pharmaceuticals for human use, notably *Rituxan* (IDEC/Genentech/Hoffman la Roche) and *Herceptin* (Genentech/Hoffman la Roche) with over 50 other projects in the research and development pipeline. Antibodies also may be employed in active immunotherapy utilising anti-idiotype antibodies which appear to mimic (in an immunological sense) cancer antigens. Although elegant in concept, the utility of antibody-based approaches may ultimately prove limited by the phenomenon of 'immunological escape,' where a subset of cancer cells in a mammalian or human subject mutates and loses the antigen recognized by the particular antibody and thereby can lead to the outgrowth of a population of cancer cells that are no longer treatable with that antibody.

Subunit vaccines

Drawing on the experience in vaccines for infectious diseases and other fields, many researchers have sought to identify antigens that are exclusively or preferentially associated with cancer cells, namely tumour specific antigens (TSA) or tumour associated antigens (TAA), and to use such antigens or fractions thereof as the basis for specific active immunotherapy.

There are numerous ways to identify proteins or peptides derived therefrom which fall into the category of TAA or TSA. For example, it is possible to utilize differential display

techniques whereby RNA expression is compared between tumour tissue and adjacent normal tissue to identify RNAs which are exclusively or preferentially expressed in the lesion. Sequencing of the RNA has identified several TAA and TSA which are expressed in that specific tissue at that specific time, but therein lies the potential deficiency of the approach in that identification of the TAA or TSA represents only a "snapshot" of the lesion at any given time which may not provide an adequate reflection of the antigenic profile in the lesion over time. Similarly a combination of cytotoxic T lymphocyte (CTL) cloning and expression-cloning of cDNA from tumour tissue has lead to identification of many TAA and TSA, particularly in melanoma. The approach suffers from the same inherent weakness as differential display techniques in that identification of only one TAA or TSA may not provide an appropriate representation of a clinically relevant antigenic profile.

Over fifty subunit vaccine approaches are in development for treating a wide range of cancers, although none has yet received marketing authorization for use as a human pharmaceutical product. In a similar manner to that described for antibody-based approaches above, subunit vaccines also may be limited by the phenomenon of immunological escape.

Gene therapy

Most gene therapy trials in humans concern cancer treatment. A substantial proportion of these trial have purported to trigger and/or amplify patients' immune responses. Of particular note in are Allovectin-7 and Leuvectin, developed by Vical Inc for a range of human tumours, and StressGen Inc.'s stress protein gene therapy for melanoma and lung cancer. It is too early to judge whether these and the other 'immuno-gene therapies' in development by commercial and academic bodies ultimately will prove successful. However the commercial utility of these approaches are expected to be more than a decade away.

Cell-based vaccines

Tumours have the remarkable ability to counteract the immune system in a variety of ways. These include, downregulating the expression of potential target proteins; mutation of potential target proteins; downregulating surface expression of receptors and other proteins; downregulating MHC class I and II expression thereby hindering direct presentation of TAA or TSA peptides; downregulating co-stimulatory molecules leading to incomplete stimulation

of T-cells and thus to anergy; shedding of selective, non representative membrane portions that act as decoys to the immune system; shedding of selective membrane portions that anergise the immune system; secreting inhibitory molecules; inducting T-cell death; and other ways. Because of this wide diversity of escape mechanisms, their immunological heterogeneity and plasticity, tumours growth has to be matched with suitable immunotherapeutic strategies that can account for such heterogeneity. The potential advantages are:

(a) whole cells contain a broad range of antigens, providing an antigenic profile of sufficient heterogeneity to match that of the lesions as described above;

(b) being multivalent (i.e. containing multiple antigens), the risk of immunological escape is reduced (the probability of cancer cells 'losing' all of these antigens is remote); and

(c) cell-based vaccines include TSAs and TAAs that have yet to be identified as such; it is possible if not likely that currently unidentified antigens may be clinically more relevant than the relatively small number of TSAs/TAAs that are known.

Cell-based vaccines fall into two categories. The first category uses autologous cells. Typically a procedure within this category begins with taking a biopsy from a patient, cultivating tumour cells from the biopsy *in vitro*, modifying the cultivated cells through transfection and/or other means, irradiating the modified cells to render them replication-incompetent, and then injecting the replication-incompetent cells back into the same patient as a vaccine. Although this approach enjoyed considerable attention over the past decade, it has been increasingly apparent that this individually-tailored therapy is inherently impractical for several reasons. The procedure is time consuming as the lead time for producing clinical doses of vaccine often may exceed the patients' life expectancy. The procedure may be expensive and, as a 'bespoke' product, it is not possible to specify a standardised product (only the procedure, not the product, can be standardised and hence optimised and quality controlled). Still further, the tumour biopsy used to prepare the autologous vaccine generally will have unique growth characteristics, interactions and communications with surrounding

tissue. The characteristics of the initial cell sample, which reflect a particular environment at a single time point from a tumour may severely limit the use of autologous cells for immunotherapy:, wherein a vaccine desirably may be administered over the entire presentation time of a disease.

The second category of cell-based vaccines utilize allogeneic cells. These vaccines comprise cells that that genetically (and hence immunologically) are mismatched to patients. Allogeneic cell procedures benefit from the same advantages of multivalency as autologous cells. In addition, allogeneic cell vaccines can utilize immortalized cell lines, which can be cultivated indefinitely *in vitro*. Thus, this approach overcomes the lead-time and cost disadvantages of autologous methodologies.

Numerous publications extol the utility of cell-based cancer vaccines. See, for example, Dranoff, G. *et al.* WO 93/06867; Gansbacher, P. WO 94/18995; Jaffee, E.M. *et al.* WO 97/24132; Mitchell, M.S. WO 90/03183; and Morton, D.M. *et al.* WO 91/06866. These studies report procedural variations that range from a basic technique of using cancer cells as an immunotherapy antigen, to transfecting the cells to produce GM-CSF, IL-2, interferons or other immunologically-active molecules to the use of 'suicide' genes. Various research groups have reported the use of allogeneic cell lines for use against melanoma, that are HLA-matched or partially-matched to a patients' haplotype and allogeneic cell lines that are mismatched to the patients' haplotype. Also described are mismatched allogeneic prostate cell lines transfected with GM-CSF.

Despite this intensive work in a crucial field of medical science, successful and reproducible eradication or inhibition of cancer growth remains elusive. Any new material or procedure that can address and at least partially overcome the limitations inherent in the use of cell based vaccines would provide very important benefits for treatment of this disease. These needs are satisfied by the present invention.

Summary of the Invention

Embodiments of the invention alleviate the problems in the field summarized above in

several ways. One embodiment provides an allogeneic immunotherapy vaccine for the treatment of prostate cancer in a patient, comprising an adjuvant, cells from a first allogeneic normal prostate cell line, cells from a second allogeneic cell line obtained from a primary prostate cancer biopsy, and cells from a third allogeneic cell line obtained from a metastasis of prostate cancer.

Another embodiment provides an allogeneic immunotherapy vaccine for the treatment of prostate cancer in a patient, comprising an adjuvant, allogeneic cells from a first normal prostate cell line, allogeneic cells from a second immortalized cell line obtained from a prostate cancer biopsy, and allogeneic cells from a third immortalized line obtained from a prostate cancer biopsy, wherein cells of the second immortalized cell line express normal levels of neutral endopeptidase but low levels of endothelin converting enzyme and cells of the third immortalized cell line express unusually low levels of both neutral endopeptidase and endothelin converting enzyme.

Other embodiments will be appreciated by a skilled artisan upon reading the specification. Many of these embodiments concern selected cell lines used in allogeneic immunotherapy agents for the treatment of cancer. These types of vaccine provided unexpected favourable clinical outcomes.

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Figure 6 shows PSA levels in non-responders increasing logarithmically.

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Figure 8 shows reduction in the rate of PSA increase for a patient.

Figure 9 shows PSA doubling time results for 15 patients.

Figure 10 shows the ratio of PSA doubling time for 15 patients.

Detailed Description

Generally, cell-based cancer vaccines until now have a common feature of employing cells that contain at least some TSAs and/or TAAs that are shared with antigens present in a patient's tumour. In each case, tumour cells are utilized as the starting point on the premise that only tumour cells will contain TSAs or TAAs of relevance, and the tissue origins of the cells are matched to the tumour site in patients.

In contrast to this expectation in the field, some embodiments of the invention utilize immortalized 'normal,' non-malignant cells as a basis of allogeneic cell cancer vaccines. Normal cells are not expected to posses TSAs or relevant concentrations of TAAs. Hence it was surprising that normal cells, and particularly combinations of normal cells with cells derived from tumour biopsies as described herein are effective as anti-cancer vaccines. The approach is general and can be adapted to any mammalian tumour by the use of immortalized normal cells derived from the same particular tissue as the tumour intended to be treated. Immortalized normal cells can be prepared by those skilled in the art using published methodologies, or they can be sourced from cell banks such as ATCC or ECACC, or they are available from several research groups in the field.

A prostate cancer vaccine, for example may include one or a combination of different immortalized normal cell lines derived from the prostate and can be prepared using methods reviewed and cited in Rhim, J.S. and Kung, H-F., 1997 Critical Reviews in Oncogenesis 8(4):305-328 or selected from PNT1A (ECACC Ref No: 95012614), PNT2 (ECACC Ref No: 95012613) or PZ-HPV-7 (ATCC Number: CRL-2221). In an embodiment, a clonal derivative of PNT-2, named OnyCap-23 desirably is combined with cells obtained from primary or metastatic cancer biopsies. Accordingly, a further embodiment is the addition of TSAs and/or TAAs by combining one or more immortalized normal cell line(s) such as OnyCap-23 with one, two three or more different cell lines derived from primary and/or metastatic cancer biopsies. In an embodiment, prostate cancer cells from at least one cell line

derived from a metastasis biopsy from lymph node, bone, brain or liver tissue are combined with at least one cell line derived from a biopsy of unmetastasised tissue from a prostate. In a particularly favourable embodiment, the metastasised sample derived cell line is LnCaP and the cell line from a primary prostate cancer biopsy is P4E6. Of course, compositions of these cell lines may further include cells or cell lines.

In another embodiment pursuant to the strategy of combining cells from a normal tissue cell line with other cells from at least two identifiable stages of cancer progression, cells from a normal prostate cell line are combined with at least one cell line representative of non-metastasised cells and at least one cell line representative of metastasised cells.

In an embodiment preferably cells from the LnCaP and P4E6 cell lines are combined with cells from a normal prostate cell line (such as OnyCap-23). The LnCaP and P4E6 cells in such formulations may be replaced or supplemented with cells from other cell lines that are derived from lymph node metastases or primary prostate cancers respectively may replace the LnCaP cells. Other cell lines representative of normal prostate cells may replace OnyCap-23, such as, for example, PNT-2 cells.

In yet another embodiment, a prostate cell line obtained from a tumour biopsy that exhibits high neutral endopeptidase-24.11 activity and low endothelin-converting enzyme activity (for example, as determined using the methods of Usmani et al, the relevant passages of which are particularly incorporated by reference) may replace the LnCaP cells. In yet another embodiment, a prostate cell line obtained from a tumour biopsy that exhibits low levels of both neutral endopeptidase-24.11 and endothelin-converting enzyme may replace the P4E6 cells. In another embodiment, other cell lines representative of normal prostate cells may replace OnyCap-23, such as, for example, PNT-2 cells.

In yet another embodiment, a vaccine comprises allogeneic cells from a first normal prostate cell line, allogeneic cells from a second immortalized cell line obtained from a prostate cancer biopsy that express normal levels of neutral endopeptidase but low levels of endothelin converting enzyme, and allogeneic cells from a third immortalized line obtained from a prostate cancer biopsy that express unusually low levels of both neutral endopeptidase

and endothelin converting enzyme. In this instance, the presence of tumour associated glycoprotein related to sialyated Tn antigen, as described by the work of Brenner et al. (see J. Urology 153: 1575-1579 1995) may be used to select a cell line obtained from a the primary prostate cancer. The differential presentation of neutral endopeptidase -24.11 and endothelin converting enzyme as described by Usmani et al. in Clin. Science 103 (supp 48): 3145-3175 can be used for this embodiment.

All the cell lines described herein will show good growth in large scale cell culture and sufficient characterization to allow for quality control and reproducible production.

Preferably, the cell lines are lethally irradiated utilizing gamma irradiation at 20-400 Gy to ensure that they are replication incompetent prior to use in the mammal or human.

The cell lines and combinations referenced herein preferably are frozen, freeze dried or otherwise stabilized to allow their transportation and storage. Accordingly, in a further embodiment a combination of cells referenced herein may be formulated with a cryoprotectant solution. Suitable cryoprotectant solutions may include but are not limited to, 10-30% v/v aqueous glycerol solution, 5-20% v/v dimethyl sulphoxide or 5-20% w/v human serum albumin may be used either as single cryoprotectants or in combination.

Cells obtained from the cell lines may be mixed in any convenient proportion. Preferably, no cell type is present in the mixture more than 20 fold (measured using total amounts of DNA) more than any other cell type. More preferably the ratio is less than 10, 5 or 3 fold.

Yet a further embodiment is the use of a cell line combination with a non-specific immune stimulant such as BCG, M. Vaccae, a Mycobacterium, Tetanus toxoid, Diphtheria toxoid, Bordetella Pertussis, interleukin 2, interleukin 12, interleukin 4, interleukin 7, Complete Freund's Adjuvant, Incomplete Freund's Adjuvant or another known non-specific agent. Such general immune stimulants advantageously can enhance immune status whilst the combinations of cell lines, contribute to immune enhancement via haplotype mismatch and, at the same time, target an immune response to a plethora of TAA and TSA due to their

heterogeneity.

Example 1

Growth, irradiation, formulation and storage of cells

An immortalised cell line derived from normal prostate tissue namely PNT2 was grown in roller bottle culture in RPMI 1640 media supplemented with 2 mM L-glutamine and 5% fetal calf serum (FCS) following recovery from liquid nitrogen stocks. Following expansion in T175 static flasks the cells were seeded into roller bottles with a growth surface area of 850 cm^2 at 1-20 $x10^7$ cells per roller bottle.

An immortalized cell line derived from primary prostate tissue namely NIH1542-CP3TX was grown in roller bottle culture in KSFM media supplemented with 25μg/ml bovine pituitary extract, 5ng/ml of epidermal growth factor, 2 mM L-glutamine, 10mM HEPES buffer and 5% fetal calf serum (FCS) (hereinafter called "modified KSFM") following recovery from liquid nitrogen stocks. Following expansion in T175 static flasks the cells were seeded into roller bottles with a growth surface area of 1,700 cm^2 at 2-5 $x10^7$ cells per roller bottle.

Two secondary derived cell lines, LnCap and Du145, obtained from ATCC were used. LnCap was grown in large surface area static flasks in RPMI media supplemented with 10% FCS and 2 mM L-glutamine following seeding at 1-10$x10^6$ cells per vessel and then grown to near confluence. Du-145 was expanded from frozen stocks in static flasks and then seeded into 850 cm^2 roller bottles at 1-20$x10^7$ cells per bottle and grown to confluence in DMEM medium containing 10% FCS and 2 mM L-glutamine. All cell lines were harvested utilising trypsin at 1x normal concentration. Following extensive washing in DMEM the cells were re-suspended at a concentration of 5-40$x10^6$ cells/ml and irradiated at 50-300 Gy using a Co^{60} source. Following irradiation the cells were formulated in cryopreservation solution composing of 10% DMSO, 8% human serum albumin in phosphate buffered saline, and frozen at a cell concentration of 5-150 $x10^6$ cells/ml, in liquid nitrogen until required for use.

Vaccination Schedule

Prostate cancer patients were selected on the basis of being refractory to hormone therapy with a serum PSA level of at least 30 ng/ml.

	Cell Lines Administered		
Dose	**Trial Arm A**	**Trial Arm B**	**Trial Arm C**
1, 2 and 3	PNT2	Du145	LnCap
4 and subsequent	PNT2 / Du145/ NIH1542	PNT2 / Du145/ LnCap	PNT2 / NIH1542/ LnCap

The cells were warmed gently in a water bath at 37°C and admixed with mycobacterial adjuvant prior to injection into patients. Injections were made intra-dermally at four injection sites into draining lymph node basins. The minimum interval between doses was two weeks, and most of the doses were given at intervals of four weeks. Prior to the first dose, and prior to some subsequent doses, the patients were tested for delayed-type hypersensitivity (DTH) against the four cell lines listed in the vaccination schedule above (all tests involved 0.8×10^6 cells with no adjuvant).

Immunological responses were analyzed. T-Cell proliferation responses were determined as follows. To evaluate the expansion of T-cell populations that recognize antigens of the vaccinating cell lines, a T cell proliferation assay was used that employed stimulation with lysates from the prostate cell lines. Whole blood was extracted at each visit to the clinic and used in a BrdU (bromodeoxyuridine) based proliferation assay as described below:

Patient BrdU proliferation method

Reagents

RPMI	Life Technologies, Paisley, Scotland
BrdU	Sigma Chemical Co, Poole, Dorset

PharMlyse	35221E	Pharmingen, Oxford UK
Cytofix/Cytoperm	2090KZ	"
Perm/Wash buffer (x10)	2091KZ	"
FITC Anti-BrdU/Dnase	340649	Becton Dickinson
PerCP Anti-CD3	347344	"
Pe Anti-CD4	30155X	Pharmingen
Pe Anti-CD8	30325X	"
FITC mu-IgG1	349041	Becton Dickinson
PerCP IgG1	349044	"
PE IgG1	340013	"

In this method, 1 ml blood is diluted with 9 ml RPMI + 2mM L-gln + PS + 50μM 2-Me. Serum should not be added. The blood is left overnight at 37°C. The following morning, 450μl of diluted blood were aliquoted into wells of a 48-well plate and 50μl of stimulator lysate added. The lysate was made by freeze-thawing tumour cells ($2x10^6$ cell equivalents/ml) x3 in liquid nitrogen and then stored aliquots frozen until required. Cells are cultured at 37°C for 5 days. On the evening of day 5 50μl BrdU at 30μg/ml are added. One hundred μl of each sample are aliquoted each into cells of a 96-well round-bottomed plate. Each plate is spun and supernatant discarded. Red cells are lysed using 100μl *Pharmlyse* for 5 minutes at room temperature, and then washed x2 with 50μl of Cytofix. The samples are spun and supernatant removed by flicking. Then the cells are permeabilized with 100μl Perm wash for 10 mins at RT. Thirty microliters of antibody mix are added, that comprise antibodies at correct dilution made up to volume with Perm-wash. The mixtures are incubated for 30 mins in the dark at room temperature. This is followed by wash x1 and resuspension in 100μl 2% paraformaldehyde. This is added to 400μl FACSFlow in cluster tubes ready for analysis. Analysis is carried out with a FACScan, and storing 3000 gated CD3 events.

The set ups of microtiter plate conditions are shown in Table 1 and Table 2.

Table 1: 6-well plate for stimulation

	Nil	ConA	1542	LnCap	Du145	Pnt2
PBL 1						
PBL 2						
PBL 3						
PBL 4						
PBL 5						
PBL 6						

Table 2: 96-well plate for antibody staining

PBL 1		PBL 2		PBL 3		PBL 4		PBL 5		PBL 6	
Nil A	15 D	Nil A	15 D	Nil A	15 D	Nil A	15 D	Nil A	15 D	Nil A	15 D
Nil D	15 E	Nil D	15 E	Nil D	15 E	Nil D	15 E	Nil D	15 E	Nil D	15 E
Nil E	Ln D	Nil E	Ln D	Nil E	Ln D	Nil E	Ln D	Nil E	Ln D	Nil E	Ln D
Con D	Ln E	Con D	Ln E	Con D	Ln E	Con D	Ln E	Con D	Ln E	Con D	Ln E
Con E	Du D	Con E	Du D	Con E	Du D	Con E	Du D	Con E	Du D	Con E	Du D
	Du E		Du E		Du E		Du E		Du E		Du E
	Pn D		Pn D		Pn D		Pn D		Pn D		Pn D
	Pn E		Pn E		Pn E		Pn E		Pn E		Pn E

Table Legend

A: IgG1-FITC (5µl) IgG1-PE (5µl) IgG1-PerCP (5µl) 15µlMoAb+15µl)

D:	BrdU-FITC (5µl) 15µlMoAb+15µl	CD4-PE (5µl)	CD3-PerCP (5µl)
E:	BrdU-FITC (5µl) 15µlMoAb+15µl	CD8-PE (5µl)	CD3-PerCP (5µl)

15: NIH1542-CP3TX

Ln: LnCap

D: Du1145

Pn: PNT2

Con: ConA lectin (positive control)

Nil: No stimulation

The results for the proliferation assays are shown in Figure 1, wherein a proliferation index for either CD4 or CD8 positive T-cells are plotted against the various cell lysates. The proliferation indexs are derived by dividing through the percentage of T-cells proliferating by the no-lysate control.

Results are shown for three patients (numbers 112, 307 and 406). Results are given for four cell lysates namely, NIH1542, LnCap, DU-145 and PNT-2. Overall, 50% of patients treated mount a specific proliferative response to at least one of the cell lines.

Western blots using patient serum were carried out. Standardized cell lysates were prepared for a number of prostate cell lines to enable loading of similar quantities of protein on a denaturing SDS PAGE gel for Western blot analysis. Each blot was loaded with

molecular weight markers, and equal amounts of protein derived from cell lysates of NIH1542, LnCap, DU-145 and PNT-2. The blot then was probed with serum from patients derived from pre-vaccination and following 16 weeks vaccination (four to six doses).

In this method, of sample preparation from prostate tumour lines, cell pellets are washed 3 times in PBS, and then re-suspended at 1 x 10^7 cells/ml of lysis buffer. The re-suspended cells are passed through 5 cycles of rapid freeze thaw lysis in a liquid nitrogen/water bath. The cells then are centrifuged at 1500 rpm for 5 minutes to remove cell debris, and ultracentrifuged at 20,000 rpm for 30 min to remove membrane contaminants. These are aliquoted at 200 µl and stored at -80°C. Gel electrophoresis is carried out by mixing lysates 1:1 with Laemelli sample buffer and boiling for 5 minutes. Then, 20 µg samples are loaded into 4-20% gradient gel wells. The sample gels are electrophoresed in Bjerrum and Schafer-Nielson transfer buffer (with SDS) at 200 V for 35 minutes.

Western transfer methods were carried out by equilibrating gels, nitrocellulose membranes and blotting paper in transfer buffer for 15 minutes. Western blot data from serum of patients 115, 307 and 406 are presented as Figure 2. Then gel-nitrocellulose sandwiches are arranged on anodes of semi-dry electrophoretic transfer cells made from 2 sheets of blotting paper, nitrocellulose membrane, gel, and 2 sheets of blotting paper. A cathode is applied and sandwiches exposed to 25 V for 90 minutes. Immunological detection of proteins was carried out by blocking nitrocellulose membranes overnight at 4°C with 5% Marvel in PBS/0/05% Tween 20. The membranes were rinsed twice in PBS/0.05% Tween 20, then wash for 20 min and 2 x 5 min at RT on a shaking platform. The membranes were then incubated in 1:20 dilution of clarified patient plasma for 120 min at RT on a shaking platform. This was followed by a wash as above with an additional 5 min final wash. The membranes were then incubated in 1:250 dilution of biotin anti-human IgG of IgM for 90 min at RT on a shaking platform, then washed as above with an additional 5 min final wash. Then the membranes are incubated in 1:1000 dilution of streptavidin-horseradish peroxidase conjugate for 60 min at RT on a shaking platform, and washed as above. The membranes are then incubated in Diaminobenzidine peroxidase substrate for 5 min to allow color development. The reaction is stopped by rinsing the membranes with water.

Figure 3 shows 3 results obtained from 3 patients (112, 305 and 402). These results show clearly that vaccination over a 16 week period, using four to six doses, can cause an increase in antibody titre against cell line lysates as well as cross reactivity against lysates not received in this vaccination regime (other than DTH testing).

Antibody titres were determined by coating ELISA plates with standardised cell line lysates and by dilution studies on serum from vaccinated patients. The Elisa method utilized anti-lysate IgG. Plates were coated with 50 µl/well lysates at 10µg/ml according to dilutions in the following Table 3.

Table 3. Dilution Table

Lysate	Protein conc	Coating conc	Amount/ml	Amount
PNT2	2.5 mg/ml	10 µg/ml	3.89 µl	19.4 µl
1542	4.8 mg/ml	10 µg/ml	2.07 µl	10.3 µl
Du145	2.4 mg/ml	10 µg/ml	4.17 µl	20.8 µl
LnCap	2.4 mg/ml	10 µg/ml	4.12 µl	20.6 µl

Each sample was covered and incubate overnight at 4°C, followed by wash x2 with PBS-Tween. Each plate was pounded on paper towels to dry and then samples blocked with PBS/10%FCS (100µl/well). These were covered and incubate at room temperature (RT) for 1 hour (minimum) and then wash x2 with PBS-Tween. Then 100µl PBS-10% FCS were added to samples in rows 2-8, 200µl plasma samples (diluted 1 in 100 in PBS-10%FCS that is, 10µl plasma added to 990µls PBS- 10% FCS) to row 1 and serial 100µl dilutions made down the plate below the row. The extra 100µl from bottom wells were discarded and each plate covered and incubated in a refrigerator overnight.

Biotinylated antibody solution (Pharmingen; IgG 34162D) was diluted and added ie. final conc 1mg/ml (ie 20ml in 10mls). The samples were covered and incubated at RT for 45 minutes and washed x6 as above. A dilute streptavidin –HRP conjugate obtained from Pharmingen, (13047E 0) was diluted 1:1000 (ie. 10ml ->10 mls) and added to 100ul/well.

The samples were incubated at 30 min at RT and then wash x8. Then 100ul substrate solution was added to each well and signal allowed to develop for 10-80 min at RT. The colour reaction was stopped by adding 100ul 1M H_2SO_4 per well and the optical densities determined at 405nm.

Results obtained indicated that, antibody titres at baseline (0), 4 weeks, 8 weeks and 16 weeks for the 3 patients (112, 305 and 402) increase. The data show that after vaccination with at least four doses, patients exhibit increased antibody titre against cell line lysates and also cross-reactivity against cell lines not received in this vaccination regime (except as DTH doses).

PSA levels were evaluated for patients receiving the vaccine at entry into the trial and throughout the course of vaccination, using routinely used clinical kits. The PSA values for three patients (110, 303 and 404) are shown in Figure 4 (vertical axis is serum PSA in ng/ml; horizontal axis is time, with the first time point representing the initiation of the vaccination programme) and portray a drop or partial stabilization of the PSA values, which in this group of patients normally continues to rise, often exponentially. The result for patient 110 is somewhat confounded by the radiotherapy treatment to alleviate bone pain, although the PSA level had dropped prior to radiotherapy.

Example 2: Use of a Normal Melanocyte in a Murine Melanoma Protection Model Model

A normal melanocyte cell line was used in a vaccination protection model of murine melanoma utilising the B16.F10 as the challenge dose. The C57 mice received two vaccinations of either PBS, $5x10^6$ irradiated K1735 allogeneic melanoma cells or $5x10^6$ irradiated Melan P1 autologous normal melanocyte cells on days -14 and -7. Challenge on day 0 was with 1 x 10^4 B16.F10 cells and tumor volume measured every three days from day 10 onwards. Animals were sacrificed when the tumor had grown to 1.5 x 1.5 cm measured across the maximum dimensions of the tumor. It was found that that vaccination with Melan1P cells offer some level of protection against this particularly aggressive murine tumour, as seen in Figure 5.

Example 3: Phase I/II Study

A phase I/II study was carried out with three types of allogeneic cells representing a normal prostate cell line, a prostate tumour derived cell line and a metastasised tumour cell line. A combined cell vaccine was given to patients having hormone refractory prostate cancer and safety, tolerability and efficacy, as measured by effect on survival and quality of life determined. The following criteria were used for inclusion of patients: patients of any age with histologically confirmed prostate cancer; patients with hormonal refractory disease following optimal first line LHRH treatment, or high dose bicalutamide (150mg per day), or orchidectomy; progressive disease indicated by a rise in serum PSA on at least 2 successive occasions separated by at least 4 weeks; serum PSA level of at least 2ng/ml at Week -2; WHO Performance Status of 0-2 at Week –4; a life expectancy of at least 6 months; the ability of the patient to read and understand the patient information leaflet and to give written informed consent; the willingness and ability of the patient to attend the hospital for all treatments and assessments; aequate bone marrow function (WBC > 3500/mm^3, haemoglobin > 9g/dl, platelet count > 100,000/mm^3 at Week 0); adequate renal function at Week 0 (serum creatinine < 2.0mg/dl); adequate hepatic function at Week 0 (< 2 times upper limit of normal, ALT 52U/l, AST 40U/l); adequate response to DTH testing with the specified intradermal antigens at Week 0; and normal 24 hour Urinary Cortisol at Week -2 (60 – 240 nmol/24 hr).

The following criteria were used for removing a patient from the study. A patient could withdraw at any time without reason. The investigator could withdraw a patient if it is in the best interest of the patient. Commencement on any other investigational agent, radiotherapy, chemotherapy or corticosteroids (e.g. in spinal cord compression) or surgical intervention was another criteria. Protocol violation by investigator or patient that in the opinion of the Sponsor's medical expert would interfere with the study was another. Unacceptable toxicity, disease progression as measured by appearance of new metastatic lesions confirmed by radiological investigations, and symptomatic disease progression also could prompt withdrawl. Patients who withdrew before completion of 6 months treatment with Ony-P1 were replaced unless withdrawal was due to disease progression or unacceptable toxicity.

An open label safety, tolerability and efficacy trial was carried out as follows. A

combined vaccine "ONY-P1" was used in a translucent plastic Cryo Sleeve that contained three Greiner Cryo.S vials, each containing $8x10^6$ irradiated cells. Each vial contained one of the following cell lines: Vial 1: LnCaP (Code CT3); Vial 2: P4E6 (Code CT4); Vial 3: OnyCap-23 (Code CT1). The vials were stored in the vapor phase of liquid nitrogen at -178°C and transported to an investigational site on the day of administration in a Dewar Flask that contains liquid nitrogen. Cells were suspended in Hanks Balanced Salts Solution plus 2% foetal calf serum plus 8% dimethyl sulfoxide. BCG was obtained by Onyvax (OncoTICE, N.V.Organon, Kloosterstraat 6, PO Box 20, 5340 BH Oss, The Netherlands, PL 05003/0046) and each dose contained 0.6-2.4 x 10^6 CFU (the range stems from the product label). The product was diluted in saline for injection to yield the specified dose in 0.1 ml. Each injection suspension contained the contents of three complete vials (one of each cell type). The total volume (made up with the requisite volume of saline for injection) was 1ml, given as 8 x 0.125 ml intradermal injections, with two injections into each draining lymph node basin. The BCG-adjuvanted doses were given at weeks 0 and 2; cells alone are given at weeks 4, 8, and at 4-weekly intervals up to and including week 48 (14 doses in total).

The study was an open label design in a maximum of 48 evaluable patients split into two cohorts. The first cohort consisted of 24 patients without bone metastases and the second consisted of 24 patients with bone metastases. The total treatment period for each individual patient was 12 months. The three-stage study was as follows: i.) stage one, a pre treatment phase and an initial treatment phase lasting four weeks in which patients receive ONY-P1 plus BCG; ii) stage two, which lasted 48 weeks wherein patients were treated once a month with ONY-P1 alone; and iii) stage three, a follow up of all patients for 12 months following completion of treatment.

The treatment-assay schedule shown in Table 4 was carried out:

Table 4 Treatment Schedule

Activity	Week Number														
	-4	-2	0	2	4	8	12	13	14	15	16	20	24	28	32
PSA (10ml)[1]	Y	Y	Y	Y	Y	Y	Y				Y	Y	Y	Y	Y
Chemistry (10ml)[2]		Y	Y	Y	Y	Y	Y				Y	Y	Y	Y	Y
Haematology (5ml)[3]		Y	Y	Y	Y	Y	Y				Y	Y	Y	Y	Y
Immunology profile	Y	Y	Y	Y	Y		Y	Y	Y	Y	Y				Y
DTH Antigens		Y									Y				

DTH Cell Lines					Y						Y				
24h urinary cortisol		Y													
Physical exam[5]	Y				Y		Y						Y		
Chest X-ray		Y					Y						Y		
Bone scan		Y											Y		
CT abdomen / pelvis		Y											Y		
EORTC QLQ-30		Y										Y			
Skin punch biopsy					Y						Y				
BCG			Y	Y											
ONY-P1			Y	Y	Y	Y	Y				Y	Y	Y	Y	Y

Activity	*33*	*34*	*35*	*36*	*40*	*44*	*48*	*49*	*50*	*51*	*52*
PSA (10ml)[1]				Y	Y	Y	Y				Y
Chemistry (10ml)[2]				Y	Y	Y	Y				Y
Haematology (5ml)[3]				Y	Y	Y	Y				Y
Immunology profile	Y	Y	Y	Y			Y	Y	Y	Y	Y
DTH Antigens						Y					Y
DTH Cell Lines						Y					
24h urinary cortisol											
Physical exam[5]				Y							Y
Chest X-ray				Y							Y
Bone scan											Y
CT abdomen / pelvis											Y
EORTC QLQ-30											Y
Skin punch biopsy						Y					
BCG											
ONY-P1				Y	Y	Y	Y				

[1] Sample to be taken in Na Heparin vacutainer. If sample taken on the same occasion as for Chemistry, then one 10 ml sample will suffice for both.

[2] Sample to be taken in Na Heparin vacutainer. If sample taken on the same occasion as for Chemistry, then one 10 ml sample will suffice for both.

[3] Sample to be taken into EDTA.

[4] Samples to be taken in Na Heparin. 10ml volume to be taken on all occasions unless written request for 50ml made by Sponsor.

[5] Including Physical/clinical assessment, vital signs and International Prostate Symptom Score.

In an initial analysis of the data, the first 15 patients who received more than 4 months of treatment were reviewed in great detail Of the 15, 5 showed statistically significant PSA velocity reductions. It was concluded that there were no safety issues, 5 of the 15 showed

stabilization of PSA titres that correlated with their immunological profile (Th1/Th2). Further analysis of data from the first 26 treated patients showed that the median time to disease progression will be in excess of 41 weeks, which compares favourably with 29 weeks for Dendreon's phase II data for its Provenge immunotherapy. The time to disease progression is clinically acceptable as a pivotal end point.

A more complete review of the final results were as follows: PSA data from the Phase II clinical trial are presented by plotting each patient on a log scale. See figures 6 through 8. Data taken prior to vaccination are shown in grey lines, which corresponds to the left side of the plot until the sustained increase in Figure 6, and the first third of the plots of figures 7 and 8 prior to levelling off. Data taken during vaccination is shown as later data points which are more levelled off. In hormone refractory prostate cancer the PSA level increases logarithmically until death. Certain existing therapies do show effects on PSA velocity, but these have been transient and not associated with effects on patient survival. At this disease stage, the PSA velocity either remains constant, or, as shown by patient 6 (non-responder) below, increases.

Figures 6 through 8 show PSA levels in individual patients, which increased logarithmically. A control, or non-responder as represented by patient results shown in Figure 6 shows a PSA level that increases logarithmically until death. The y axis depicts ln PSA concentration and the x axis depicts time over a year period. This figure shows a 63 percent decreased PSA velocity from vaccination. The data to the left of the inflection mark show velocity prior to treatment and the data points to the right show the lower velocity after treatment. The straight lines from left to right indicate the linear regression of the data before and after treatment, respectively.

Some typical existing therapies show effects on the "PSA velocity" as seen in the dip in the plot of this figure, but such effects generally are transient and not associated with effects on patient survival. In contrast, five of the first 15 patients analysed, sustained statistically significant reductions in PSA velocity (e.g. patients 1 and 14), the data of which are shown in Figures 7 and 8. More importantly, a decrease in velocity of PSA was seen

equivalent to an increase in PSA doubling time, as summarized in figures 9 and 10. Figure 9 provides representative data from 15 patients and Figure 10 depicts the ratio of PSA doubling times before and after treatment.

Further clinical data were obtained. The following immunological assays were carried out on patients' blood: whole blood proliferation, Cytometric Bead Array, qPCR for cytokines, cell surface marker analysis, and T-cell cloning and tetramers. Correlations were found between PSA responses and assay results. In a review of 26 patients, 5 have shown disease progression and 21 remain progression free. If a further 8 were to show disease progression simultaneously on the last reporting day, the median time to disease progression would be 41 weeks.

It is to be understood that while the invention has been described in detail by way of example and illustration for the purpose of clarity of teaching, the foregoing description is not intended to limit the scope of the invention. Other aspects, advantages, and modifications that are apparent to one of skill in the art in light of the teachings of this invention are within the scope of the following claims.

Each document cited herein is incorporated by reference in its entirety. Great Britain patent application No. 9827103.4, filed December 10, 1998, PCT/GB99/04135, filed December 9, 1999 and U.S. patent application No. 09/857,690, filed June 8, 2001 are incorporated in their entireties by reference.